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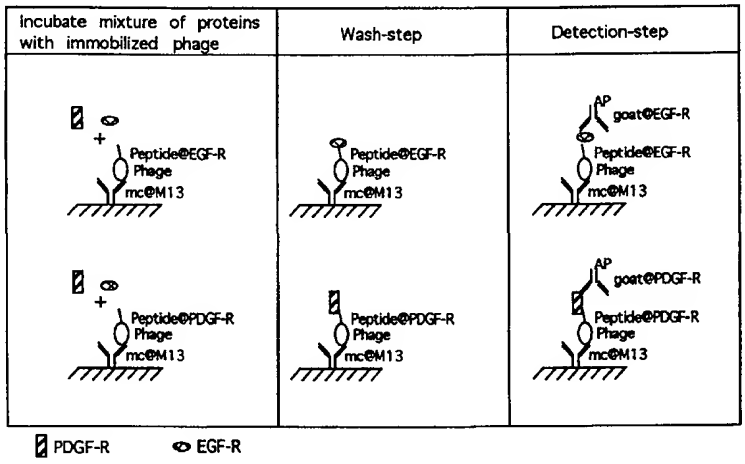

(57) Abstract

A method of using antibody expression clones (e.g. bacteria, bactenophage expressing single chain antibodies) bound to a solid support for identifying genes which are differentially expressed between two cell types is described. The invention can be used to identify proteins and genes which are differentially expressed between any two cells, for example diseased and non–diseased cells, cells exposed to an environmental factor or an exogenous substance and those not exposed, cells of different organs or organisms, or cells at different stages of a developing organism. Antibody expression clones can also be used to identify intracellular proteins which are involved in cellular responses.

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SUBTRACTIVE PROTEIN SCREENING FOR GENE IDENTIFICATION

FIELD OF THE INVENTION

The invention relates to the area of gene expression. More particularly, the invention relates to the area of identifying genes which are differentially expressed between two cells.

BACKGROUND OF THE INVENTION

Phenotypic differences between tissues are attributable to differential gene expression. For example, neoplastic or other diseased cells express genes which are not expressed by non-diseased cells, and these expressed genes contribute to the diseased phenotype. Identification of such genes would provide the art with tools to manipulate their expression so as to control a particular disease state.

Current methods for identifying differentially expressed genes relies on RNA substraction or differential display of mRNA (Lee *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:2825-2829). Substraction methods are sensitive and detect somewhat rare mRNAs. However, the method recovers genes incompletely and selects for genes in only one direction at a time during two way comparisons between cells.

Differential display can be used to provide a picture of mRNA composition of cells. A powerful application of differential display is the ability to clone differentially expressed RNAs in differing biological systems. Due drawback of the method is the problem of false-positive differences. Furthermore, there is no distinction between mRNA molecules which encode proteins. Thus, there is a need in the art for methods of identifying differentially expressed proteins.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for identifying an antibody which binds to a differentially expressed protein.

It is another object of the invention to provide a method for identifying a gene which is differentially expressed between two cells.

It is yet another object of the invention to provide a solid support comprising a pattern of antibody expression clones.

It is still another object of the invention to provide a kit for identifying a gene which is differentially expressed between a first cell and a second cell.

It is even another object of the invention to provide a method of identifying a gene which encodes an intracellular protein which participates in a cellular response.

These and other objects of the invention are provided by one or more of the embodiments described below.

One embodiment of the invention provides a method for identifying an antibody which binds to a differentially expressed protein. Labeled proteins from a first cell are contacted with a first solid support under conditions where antibodies specifically bind to proteins. The first solid support comprises a pattern of antibody expression clones, wherein the antibody expression clones express fusion proteins comprising single chain antibodies. The single chain antibodies are immunoreactive with proteins of the first cell. A first pattern of immunoreactivity on the first solid support formed by the binding of labeled proteins from the first cell with the single chain antibodies expressed by the antibody expression clones is determined. Labeled proteins from a second cell are contacted with a second solid support. The second solid support comprises the pattern of antibody expression clones. A second pattern of immunoreactivity on the second solid support formed by the binding of labeled proteins from the second cell with the single chain antibodies expressed by the antibody expression clones is determined. The first pattern of immunoreactivity is compared with the second pattern of immunoreactivity. A difference between the first and second patterns of immunoreactivity identifies an antibody which binds to a protein which is differentially expressed between the first cell and the second cell.

Another embodiment of the invention provides a method for identifying a gene which is differentially expressed between two cells. A single chain antibody which binds to a protein which is differentially expressed between two cells is identified as described above. The identified single chain antibody is isolated and used to screen a population of protein expression clones comprising cDNA obtained

by reverse transcription of mRNA isolated from the first cell. A protein expression clone which binds to the single chain antibody is identified as expressing a cDNA encoding a protein which is differentially expressed between the two cells.

Yet another embodiment of the invention provides a solid support comprising a pattern of antibody expression clones. The antibody expression clones express fusion proteins comprising single chain antibodies.

Still another embodiment of the invention provides a kit for identifying a gene which is differentially expressed between a first cell and a second cell. The kit comprises a first solid support comprising a pattern of antibody expression clones. The antibody expression clones express fusion proteins comprising single chain antibodies, wherein the single chain antibodies are immunoreactive with proteins of the first cell. The first solid support can be used to identify a single chain antibody which binds to a protein which is differentially expressed between a first cell and a second cell. The kit also comprises a second solid support comprising a pattern of protein expression clones. The protein expression clones comprise cDNA obtained by reverse transcription of mRNA isolated from the first cell. The protein expression clones express cDNA encoding proteins of the first cell. The second solid support can be screened with the identified single chain antibody to identify a protein expression clone which expresses a cDNA encoding a protein which is differentially expressed between the first cell and the second cell.

Even another embodiment of the invention provides a method of identifying a gene which encodes an intracellular protein which participates in a cellular response. A library of nucleic acid vectors encoding single chain antibodies is introduced into a population of cells. The vectors express single chain antibodies intracellularly, and the single chain antibodies bind to intracellular proteins of the population of cells. The population of cells is contacted with an inducer of a cellular response. The inducer induces the cellular response in a first subset of the population of cells but does not induce the cellular response in a second subset of the population of cells. At least one nucleic acid vector encoding a single chain antibody is isolated from the second subset of the population of cells. The single chain antibody is expressed and used to screen a population of protein expression clones. The population of protein expression clones comprises cDNA obtained by

reverse transcription of mRNA isolated from the population of cells. A protein expression clone which binds to a single chain antibody is identified as expressing a cDNA encoding an intracellular protein which is involved in the cellular response in the population of cells.

The present invention thus provides the art with methods of using clones which express single-chain antibodies to identify both proteins and genes which are differentially expressed between two cells. The two cells can be any two cells, for example diseased and non-diseased cells, cells exposed to an environmental factor or an exogenous substance and those not exposed, cells of different organs or organisms, or cells at different stages of a developing organism.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts an ELISA experiment using specific immobilized peptide-phages to capture specific target protein receptors.

DETAILED DESCRIPTION OF THE INVENTION

Compositions and methods for identifying differentially expressed proteins and genes are provided. The compositions comprise antibody populations which may be bound to a solid support. For purposes of the invention "antibodies" includes polyclonal or monoclonal antibodies, single chain antibodies, intrabodies, antibody fragments including Fab' and $F(ab')_2$ antibody fragments, and the like. While the invention is generally discussed in terms of single chain antibodies it is recognized that the methods of the invention can be practiced with intrabodies, Fab fragments and the like.

It is a discovery of the present invention that antibody expression clones which express single chain antibodies can be used advantageously to identify proteins and genes which are differentially expressed between two cells. Immobilizing the antibody expression clones on a solid support greatly reduces non-specific background problems which plague method using antibody expression clones in solution. Comparing proteins rather than mRNA also produces more reliable data on protein expression, since not all mRNA is translated into protein. Thus, when a differentially expressed protein is identified using the present

invention, it is likely that the identified protein is important to generating differences seen between the compared cells.

Proteins or genes which are differentially expressed between two cells are proteins or genes which are expressed in a first cell and not in a second cell to which the first cell is compared. Differential protein or gene expression can be identified between any two cells. For example, differential protein or gene expression can be identified between a cell which is diseased and a cell which is not diseased. The cell which is not diseased can be any cell which is functionally and morphologically normal, having no obvious genetic, functional, or morphological alterations. The diseased cell can be a neoplastic cell, for example, from a tumor of the breast, colon, stomach, brain, liver, lung, pancreas, reproductive system, or skin. The neoplastic cell can be a cell which has a high probability of metastasizing, such as a melanoma cell (a highly metastatic cell), or one which has a low probability of metastasizing, such as a basal cell carcinoma of the skin (a weakly metastatic cell). The diseased cell can be infected with a pathogen, such as a virus, bacterium, fungus, mycoplasma, protozoan, or prion. A cell which has been exposed to an environmental factor, such as a particular temperature, atmospheric pressure or composition, or gravitational or magnetic field, can be compared with a cell which has not been exposed to the environmental factor. A cell which has been contacted by an exogenous substance, such as a nutrient, toxin, therapeutic agent, or other chemical, can be compared with a cell which has not been contacted with the exogenous substance. It is desirable that the cells compared be as similar as possible, but for the single distinguishing property being studied.

Differential protein or gene expression between cells at different stages of development can also be identified by this method, by comparing a pattern of protein or gene expression in a cell at one developmental stage with a pattern of gene expression in a cell at a different developmental stage. The proteins or genes which are differentially expressed between any of germ cells (oocytes and spermatocytes), embryonic cells (such as cells of a morula, bastula, gastrula, or the neural crest), differentiating fetal cells, juvenile cells (cells at any developmental stage between birth and adulthood), or adult cells can be so identified.

The cells being compared can originate from any tissue of a human, such as brain, liver, skeletal muscle, heart, pancreas, spleen, blood, skin, intestine, stomach, kidney, bladder, thymus, lung, or mammary gland. Freshly isolated cells or cell lines, such as PC12, HCT116, SW480, or HeLa, can be examined for differential protein or gene expression using the present invention. Differential protein or gene expression in cells of other organisms, both vertebrates and invertebrates, can be identified. Similarly, genes or proteins which are differentially expressed in bacterial or yeast cells, for example at different stages of growth, after growth in different media, or after various chemical treatments, can also be identified. Species-specific gene or protein expression can be identified by suing the method of the present invention to compare gene or protein expression in a cell originating from one species with gene or protein expression in a cell originating from a different species. For example, genes or proteins which are expressed in bacterial but not in yeast can be so identified. Genes or proteins which are differentially expressed between two plant cells can also be identified.

The method uses antibodies to identify differentially expressed proteins. Such antibodies can be produced by any method in the art. See, for example, U.S. Patent Nos. 5,565,332; 4,946,778; 5,648,237; 5,667,988; 4,816,567; Haseman *et al.* (1990) *Proc. Natl. Acad. Sci. USA 87*:3942-3946; Huse *et al.* (1989) *Science 246*:1275-1281; McCafferty *et al.* (1990) *Nature 348*:552-554; Horwitz *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:8678-8682; Cabilly *et al.* (1984) *Proc. Natl. Acad. Sci. USA 81*:3273-3277; Horwitz (1989) *Methods in Enzymology 178*:476-496; and Condra *et al.* (1990) *J. Biol. Chem. 265*:2292-2295; the disclosures of which are herein incorporated by reference.

Antibody expression clones express fusion proteins comprising single chain antibodies. Use of such clones for expressing single chain antibodies is taught, for example, in G.P. Smith *et al.* (1985) *Science 228*:1315; S.F. Parmley & G.P. Smith *et al.* (1988) *Gene 73*:305; Soderlind *et al.* (1995) *Gene 160*:269; Calcutt *et al.* (1993) *Gene 137*:77; Pilkington *et al.* (1996) *Mol. Immunol. 33*:4399; Yamanaka *et al.* (1996) *J. Immunol. 157*:1156; and Krebber *et al.* (1997) *J. Immunol. Meth. 201*:35, which are herein incorporated by reference. The clones can be, for example, bacteriophage or phagemids which have been genetically engineered so as

to express the single chain antibodies. Phagemids are plasmids which contain an origin of expression derived from a filamentous bacteriophage (see Sambrook *et al.* (1989) *Molecular Cloning*, pp. 4.17-4.20). Intracellularly expressed antibodies are referred to as "intrabodies." See, for example, Chen *et al.* (1994) *Hum. Gene. Ther. 5*:595-601; Marasco *et al.* (1995) *Immunotechnology 1*:1-19; Rondon *et al.* (1997) *Annu. Rev. Microbiol. 51*:257-283; and von Mehren *et al.* (1996) *Curr. Opin. Oncol. 8*:493-498; herein incorporated by reference. The antibody expression clones can also be bacteria which express single chain antibodies. The clones are constructed using cDNA encoding single chain antibodies which are immunoreactive with proteins of one of the two cells to be compared. The single chain antibodies are encoded by recombinant cDNA molecules which are constructed as follows.

Whole cells or cell lysates of one of the two cell types are used to immunize an animal such as a mouse, rabbit, goat, pig, or other animal, using standard techniques known in the art. Immunization protocols can be used which will enrich the population of B cells for those cells secreting antibodies which are immunoreactive with particular cell types. These protocols enrich the number of antibodies which bind to antigens of interest and also reduces non-specific background binding in the present invention. For example, one can select for B cells which secrete antibodies immunoreactive with cells having a high probability of metastasizing (for example, melanoma cells) but not with cells having a low probability of metastasizing (for example, weakly metastatic cells such as basal cell carcinomas of the skin). In one such protocol an animal, such as a mouse, is injected prenatally with weakly metastatic human cancer cells. Thereafter, the developing mouse recognizes antigens of the weakly metastatic cells as "self." Three to four weeks later, the same mouse is injected with highly metastatic human cancer cells. The mouse will preferentially generate B cells which react with antigens of the highly metastatic cells and which are not found on the weakly metastatic cells.

Alternatively, an adult animal can be injected with weakly metastatic human cancer cells. The animal is then given a drug, for example an alkylating agent such as cyclophosphamide, chlorambucil, busulfan, melphalan, β-propriolactone,

dimethylsulfate, or diepoxybutane, to kill proliferating B cells. This procedure can be repeated two or three times. The animal is then injected with highly metastatic human cancer cells. This process enriches the number of B cells which secrete antibodies immunoreactive with highly metastatic cells, while reducing the number of other B cells present in the animal under normal conditions. Similar procedures can be used to enrich B cell populations for cells which preferentially secrete antibodies against other cell types.

Additionally, the B cells generated using any of these methods can be sorted after isolation from the immunized animal, for example by FACS sorting to separate B cells with surface markers which indicate activated B cells generated by the recent immunization from other B cells.

B cells from the immunized animal can be isolated and used to form hybridomas using techniques known in the art. Messenger RNA is purified using standard techniques from either the B cells or the resulting hybridomas. Reverse transcriptase is then used to generate first strand cDNA from the total isolated mRNA. Those of skill in the art can select suitable primers from sequences of the constant regions flanking the antibody heavy and light chain variable regions and use the primers to amplify these regions from the cDNA using the polymerase chain reaction (PCR). Complementary DNA molecules encoding single chain antibodies are then constructed by ligating a cDNA molecule encoding a variable heavy chain to a cDNA encoding a variable light chain using a linker sequence. PCR amplification is carried out on the cDNA using primers preferably containing restriction sites at their 5′ ends. The final cDNA constructs, which encode single chain antibodies immunoreactive with protein antigens from the immunizing cell type can be used to construct antibody expression clones which will express fusion proteins comprising the single chain antibodies.

The single chain antibodies to be expressed by the antibody expression clones can also be constructed synthetically, for example, by starting with a cDNA molecule encoding a constant framework region of an antibody. Oligonucleotide sequences which encode all possible combinations of peptides 6-15 amino acids in length can be synthesized using standard methods. These oligonucleotides can be combined with the constant framework region to form single chain antibodies

which will recognize epitopes which may not be recognized by the single chain antibodies derived from mRNA of an immunized animal.

In one preferred embodiment, bacteriophage or phagemids are used to produce antibody expression clones. Bacteriophage or phagemids of the antibody expression clones used in the present invention preferably express fusion proteins which comprise single chain antibodies fused to a bacteriophage surface protein. Standard recombinant DNA techniques can be used to construct cDNA molecules which encode this fusion protein, for example by ligating cDNA encoding a single chain antibody with a DNA encoding a phage surface protein such as the gene III product of the bacteriophage M13. Preferably, the cDNA encoding the single chain antibody is less than 335 base pairs in length. The DNA encoding the fusion protein is cloned into the bacteriophage or phagemid using techniques known in the art (see, for example, WO 92/01047, incorporated herein by reference). Infected bacteria are plated on agar containing the appropriate nutrient mixture for the *E. coli* strain and allowed to secrete the bacteriophage or phagemids, forming plaques. Alternatively, bacteria can be transfected with expression constructs encoding single chain antibodies by standard methods and used to form colonies of antibody expression clones.

Antibody expression clones in the plaques can be transferred to a solid support, including, but not limited to, filter membranes (such as nitrocellulose, nylon, or paper), glass or plastic slides or tissue culture plates, beads (such as latex, polyvinylchloride, or polystyrene beads), natural or synthetic fabrics, or silicon-based supports. After transfer, the solid support contains a pattern of antibody expression clones which express single chain antibodies which ar immunoreactive with proteins in one of the cells to be compared.

The proteins to be labeled and compared can be obtained from freshly isolated tissues or from cell lines. Any label known in the art can be used, including but not limited to radioisotopes and biotinylated labels. The proteins can be labeled *in vitro* or, in the case of experimental organisms other than humans, *in vivo*. Proteins are labeled *in vitro*, for example, by exposing cells to labeled amino acids under culture conditions whereby the cells incorporate the labeled amino acids into proteins. Such methods are widely known in the art. Alternatively,

experimental animals can be given food containing labeled amino acids. The labeled amino acids are incorporated into proteins. The cells containing the labeled proteins can be lysed by any means known in the art, such as osmotic shock, sonification, use of a French press or tissue homogenizer, blending with glass beads, or freezing the cells with liquid nitrogen followed by blending. Preparations of labeled proteins from the lysed cells can be obtained using standard biochemical methods.

A labeled protein preparation from a first cell can be contacted with a solid support comprising the pattern of antibody expression clones. The contacting step is carried out under conditions where the single chain antibodies expressed by the antibody expression clones will bind specifically to protein antigens in the protein preparations, for example in the presence of suitable blocking serum. A first pattern of immunoreactivity is determined. The first pattern of immunoreactivity results from the binding of labeled proteins from the first cell with the single chain antibodies of the antibody expression clones. After the contacting step, the solid support can be treated to remove unbound or non-specifically bound proteins. For example, the solid support can be rinsed one or more times in a buffer which does not contain proteins.

Plaques or colonies which contain labeled proteins bound to single chain antibodies can be visualized by methods known in the art suited for detecting the particular protein label being used. For example, radiolabeled proteins bound to the single chain antibodies can be visualized by exposure to X-ray film. Biotinylated proteins can be visualized by incubation with a streptavidin conjugate with subsequent exposure to a detection system. Antibody expression clones comprising single chain antibodies which have bound labeled proteins specifically display a signal at least two-, five-, ten-, twenty-, or fifty-fold higher than those to which labeled proteins have bound non-specifically. After treating to visualize the bound proteins, the solid support displays a pattern of immunoreactivity, indicating the location of antibody expression clones which express single chain antibodies which are bound to the labeled proteins. If desired, the pattern of immunoreactivity can be photographed or scanned into a computer to facilitate

comparison with a pattern of immunoreactivity of labeled proteins of the second cell.

A labeled protein preparation from the second cell, to which proteins in the first cell will be compared, can be contacted with a second solid support which comprises a pattern of antibody expression clones which is identical to the pattern of antibody expression clones on the first solid support. Alternatively, the first solid support can be treated to remove the bound labeled proteins of the first cell, for example by washing with a high salt buffer. Labeled proteins of the second cell can then be contacted with the treated support. A second pattern of immunoreactivity can then be determined and visualized, as described above.

The patterns of immunoreactivity from the first and second cells can be compared, for example, by visual observation or computer-assisted comparison of the solid supports themselves, or photographs or computer-generated images of the supports. A differentially expressed protein will be identified by the presence or absence of a labeled spot in one of the patterns of immunoreactivity. The labeled spot corresponds to an antibody expression clone which expresses a single chain antibody. The single chain antibody is identified as binding to a protein which is differentially expressed between the two cells that were compared.

In one embodiment, the solid support is a filter, such as a nitrocellulose or nylon filter. Colonies or plaques of antibody expression clones can be transferred from plates containing the growing clones to the filters using methods known in the art. Preferably, duplicate filter transfers are performed, and the filters are marked so they can be aligned for comparison after contacting preparations of labeled proteins from the first and second cells. Individual plaques or colonies identified as containing a clone of interest can be selected by reference to their position on the filter and the corresponding position on the plate containing the growing clones. Bacteriophage or bacteria in the plaque or colony can then be isolated and cultured, and the single chain antibody of interest can be retrieved form the culture using standard methods.

In another embodiment, the solid support comprises a pattern of beads. The beads can be any beads to which proteins or nucleic acids can be attached, including, but not limited to, latex, polystyrene, or polyvinylcholoride beads. The

solid support can be, for example, a glass or plastic slide or tissue culture plate, such as a 6-, 12-, 24-, 48-, or 96-well plate. The beads can be attached to the solid support by using an adhesive, as is known in the art. Alternatively, the beads can be placed without attachment in compartments on the solid support.

Antibody expression clones which express single chain antibodies, such as bacteriophage, can be bound to the beads using a stable but reversible chemical linkage, *e.g.*, a disulfide bond, between a protein on the surface of the antibody expression clone and the surface of the bead. Cross-linkers with spacer arms of varying lengths, such as 1-(*p*-azidosalicylamido)-4-(iodoacetamido)butane. 1-ethyl-3-(3-dimethylaminopropyl)carbodimide hydrochloride, *N*-hydroxylsulfosuccinimidyl 4-azidobenzoate sulfosuccinimidyl 4-(*p*-azidophenyl)butyrate, sulfosuccinimidyl β-[alpha-methyl-alpha-(2-pyridyldithio)toluamido]hexanoate, 1,4-di-[3′-(d'-pyridyldithio)propionamido]butane, sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide) ethyl-1,3′-dithiopriopionate, or sulfo-succinimidyl 7-azido-4-methylcoumarin-3-acetate, can also be used to attach single chain antibodies to a bead. The bead can optionally be coated with a substance such as laminin, polylysine or polyornithine, to facilitate such linkage. The beads can be arrayed in an identifiable pattern on the solid support. Identifying labels, such as unique oligonucleotide sequences, can be attached to the beads by a chemical linkage. Each oligonucleotide label can contain a unique primer site for sequencing, so that such labels can be sequenced and identified.

After identifying a bead or beads bound to single chain antibodies which bind proteins which are differentially expressed between the two compared cells, the single chain antibodies can be removed from the beads by breaking the reversible chemical linkages by which they were attached. Alternatively, they can be retrieved from a separately studied sample.

The single chain antibody corresponding, for example, to a labeled plaque, colony or identified bead, can be isolated and used to screen a population of protein expression clones which express cDNA of the first cell. To construct the protein expression clones, total mRNA is isolated from the first cell using methods known in the art. Reverse transcriptase can be used to transcribe the isolated mRNA into cDNA. The cDNA thus obtained by reverse transcription of the

isolated mRNA encodes proteins which are expressed by the first cell. These proteins include those which are differentially expressed between the first cell and the second cell.

Using standard recombinant DNA techniques, the cDNA from the first cell can be introduced into a cloning vector to form protein expression clones. A variety of cloning vectors for use with protein expression systems are available in the art, including but not limited to bacteriophage for use in bacterial expression systems, retroviral vectors for use in mammalian expression systems, or baculoviral vectors for use in insect expression systems. In a preferred embodiment, the cloning vector is a λgt11 bacteriophage and the host cell is an *E. coli* Y1090 or BNN97 cell.

Host cells comprising the cDNA-containing cloning vectors can be plated onto a suitable medium, forming protein expression clones. The protein expression clones can be allowed to form plaques or colonies, which can be transferred to a solid support. In a preferred embodiment, the solid support is a nylon or nitrocellulose filter. After the transfer, the solid support contains a pattern of protein expression clones which contain cDNA molecules which encode proteins of the first cell.

The identified single chain antibody can be labeled, for example with a radioisotopic, fluorescent, or enzymatic label, and contacted with the solid support comprising the pattern of protein expression clones. The step of contacting is carried out under conditions where the single chain antibody will bind specifically to a protein expressed from a cDNA molecule which is localized in a particular plaque on the solid support. Single chain antibodies which bind proteins specifically display a signal at least two-, five-, ten-, twenty-, or fifty-fold higher than those to which labeled proteins have bound non-specifically. Plaques or colonies containing bound labeled proteins are detected as described above. Labeled plaques or colonies are identified as containing a protein expression clone which contains DNA from a gene which is differentially expressed between the first and the second cell. That DNA can be sequenced and, if desired, used to express the protein which is differentially expressed. Genomic DNA encoding the protein can also be isolated using the DNA of the protein expression clone.

Solid supports comprising a pattern of antibody expression clones according to the present invention are also provided in a kit. As described above, the solid supports may be, for example, filter membranes (such as nitrocellulose, nylon, or paper), glass or plastic slides or tissue culture plates, beads (such as glass, sepharose, cellulose, latex, polyvinylchloride, or polystyrene beads), natural or synthetic fabrics, or silicon-based supports. The antibody expression clones express fusion proteins comprising single chain antibodies against proteins of a cell population. Preferably, the single chain antibodies are immunoreactive with proteins in a non-diseased cell. The proteins can be obtained from any mammalian cell or tissue, such as mammary gland, colon, stomach, brain, liver, lung, pancreas, reproductive system, skin, thymus, skeletal muscle, peripheral blood lymphocytes, placenta, kidney, lymph node, or prostate. The cell can be at any developmental stage, including embryonic, fetal, juvenile, or adult stages. Preferably, the kit also contains a viable sample of the cell population which was used to obtain the antibodies from which the singe chain antibodies were derived. This sample can be used for isolating proteins which are expressed in that cell population but not in the population of cells which were tested using the kit.

A kit is also provided which contains a first solid support which can be used to identify a single chain antibody which binds to a protein which is differentially expressed between a first cell and a second cell, as described above, and a second solid support comprising a pattern of protein expression clones comprising cDNA obtained by reverse transcription of mRNA isolated from the first cell. The first solid support can be used to identify a single chain antibody which binds to a protein which is differentially expressed between the first cell and a second cell. The second solid support can be screened with the identified single chain antibody to identify a protein expression clone which expresses a cDNA encoding a protein which is differentially expressed between the first cell and the second cell.

If single chain antibodies are expressed intracellularly in a population of cells to be studied, rather than on the surface of an antibody expression clone, a gene which encodes an intracellular protein which participates in a cellular response can be identified. The cellular response can be any response in which there is a

demonstrated interest. These responses include but are not limited to apoptosis, resistance to a pathogen or toxin, alterations in cellular growth, division, or differentiation, motility, chemotaxis, immune response, cell-cell adhesion, or cellular migration.

Animals can be immunized against a lysate of the cell population to be tested, as described above, and B cells expressing antibodies against intracellular proteins in the lysed cells can be obtained. Messenger RNA can be isolated from the B cells and used to construct cDNA molecules which encode single chain antibodies which are immunoreactive with protein antigens of the cell population to be tested. Nucleic acid vectors comprising the cDNA encoding the single chain antibodies can be made using standard recombinant DNA techniques. A variety of nucleic acid vectors are available for this purpose, including but not limited to retroviral and adenoviral vectors for use in mammalian cells, baculoviral vectors for use in insect cells, and bacteriophage vectors for use in bacteria. The vectors can be introduced into the population of cells to be tested using methods known in the art.

The cells to be tested can be any cells capable of being maintained *in vitro*, such as bacteria or cell lines or explant cultures of animal or plant tissues. After their introduction into the cells, the nucleic acid vectors express the single chain antibodies intracellularly, where the single chain antibodies can bind to intracellular proteins of the cells.

The population of cells containing the single chain antibody expression vectors can be contacted with an inducer of a cellular response. For example, the inducer can be Fas ligand and the cellular response can be apoptosis. The inducer can be a pathogen, such as a bacteria, virus, fungus, mycoplasma, protozoan, or prion, and the response can be resistance to the pathogen. The inducer can be a naturally occurring or a synthetic chemical, and the cellular response can be, for example, an alteration in the growth, differentiation, or immune response of the cell.

Intracellular proteins can be identified which participate in a cellular response of a diseased cell to a therapeutic chemical, for example a reduction in growth rate of a neoplastic cell. The inducer induces the cellular response in a first

subset of the population of cells. However, in a second subset of the population of cells, the cellular response is not induced because one of the single chain antibodies has bound to an intracellular protein which participates in the cellular response.

The first and the second subset of cells can be separated on the basis of an appropriate characteristic for the cellular response being examined. Separation can be based on morphology, differential adherence to the culture surface, lytic response to a pathogen, or a variety of other characteristics which will be clear to those skilled in the art based on the desired cellular response. The second subset of the population of cells can be lysed by any means known in the art, as described above. At least one nucleic acid vector encoding a single chain antibody can then be isolated from the second subset of the population of cells and used to express the single chain antibody it encodes. Such isolation and expression can be accomplished using standard techniques.

The single chain antibody can be labeled and used to screen a population of protein expression clones. Preferably the antibody is labeled, although it can be detected otherwise, *e.g.*, using a second antibody. The population of expression clones contain cDNA obtained by reverse transcription of mRNA isolated from the same type of cells. A protein expression clone which directs expression of an intracellular protein which binds to a single chain antibody is identified as encoding an intracellular protein which is involved in the cellular response in the population of cells.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Preparation of Antibody Libraries

To prepare a library of human-derived antibody genes, synthetic consensus sequences that cover the structural repertoire of antibodies encoded in the human genome can be utilized. See, for example, PCT/EP 96/03647, herein incorporated by reference. The method utilizes a single consensus antibody gene as a universal framework for highly diverse antibody libraries. The method provides for the

creation of libraries of human antibodies, wherein said polypeptides are derived from heavy or light chain variable regions wherein said structural sub-elements are framework regions, 1, 2, 3, or 4 are complementary determining regions 1, 2, or 3.

Alternatively, diverse libraries of immunoglobulin heavy (V_H) and light (V_K and V_λ) chain variable (V) genes are prepared from peripheral blood, lymphocytes of unimmunized donors by polymerase chain reaction amplification. Genes encoding single chain Sv fragments are made by randomly combining heavy and light chain V-genes using PCR, and the combinatorial library is cloned for display on the surface of a phage. See, for example, U.S. Patent No. 5,565,332, and PCT Application WO 92/01047, herein incorporated by reference.

The use of standard phage display systems for optimizing antibodies for a given target protein are known in the art. See, for example, Krabber 91997) *J. Immunol. Meth.* 201:35-55; Wamanaka, *et al.* (1996) *J. Immunol.* 157:1156-1162; Tsurushita, *et al.* (1996) *Gene* 172:59-63; Pilkington, *et al.* (1996) *Mol. Immunol.* 33:439-450; Merz, *et al.*, (1995) *J. NeuroScience Meth.* 62:213-219; the disclosures of which are herein incorporated by reference.

Plating and Transferring BacterioPhage Libraries

Bacteriophage are plated onto agar plates at high density. The recombinant phage is mixed with plating bacteria in a culture tube and incubated 20 minutes at 37°C. (In Quest and Sternberg (1979) *Meth. Enzymol.* 68:281-298; and Stent, G. S. (1971) *Molecular Genetics: An Introductory Narrative*, W. H. Freeman, NY). 0.7% top agarose is added to the culture tube and the mixture transferred to LB plates. The bacteria and agarose is dispersed onto plates (the top agarose is melted and cooled to 45° to 50°C) before use. Plates are incubated at 37°C until plaques cover the plate but are not confluent. Incubation time may vary between 6 and 12 hours depending on the type of phage and bacteria used. Plates can then be stored at 4°C.

Nitrocellulose filters are labeled with a ballpoint pen and applied face down on cold LB plates bearing bacteriophage plaques as described above. The filters are left on the plates for about five minutes to allow transfer of phage particles to

the filter. During the transfer period, the orientation of the filter to the plate is recorded by, for example, stabbing a needle through the filter into the agar at several asymmetric points around the edge of the plate. At least two replicas are made from each plate. The filters are slowly removed from the plates with blunt, flat forceps and placed face up on paper towels or filter paper. The filters are allowed to dry for at least 10 minutes. The drying process binds the plaques to the filter.

When nitrocellulose is applied to the agarose, phage particles and unpackaged DNA absorbed to the filter to produce a replica of the plate surface. If the agarose surface is not accessibly wet, there will be little spreading of the phage on the filter. After binding to the filter, the filter is treated with sodium hydroxide which destroys the phage particles and denatures the phage DNA which then binds to the nitrocellulose. The filters are placed on three MM paper and saturated with 0.2M Na OH/1.5M NaCl. Place filters on the paper face up for 1 to 2 minutes. Filters are transferred to 3 MM paper saturated with 0.4M Tris-Cl pH 7.6/2x SSC for 1 to 2 minutes and then to 3 MM paper saturated with 2x SSC for 1 to 2 minutes. Filters are dried in a vacuum oven for two hours at 80°C.

Screening of Expression Libraries

A single colony of *E. coli* strain Y1090 *hsdR*, is used as an inoculum to prepare a plating culture. The number of plates that will need to be screened can be calculated by $2x10^4$ plaques per 90-MM plate or $5x10^4$ plaques per 150-MM plate. A set of sterile tubes is arranged in a rack. In each tube, 0.1ml of the plating bacteria is mixed with 0.1ml of SM containing $3x10^4$ pfu (90-MM plates) of the bacteriophage λexpression library. The infected bacteria is incubated for 20 minutes at 37°C. 2.5ml of molten top agarose is added to each tube and the mixture poured onto LB agar plates. The infected plates are incubated for 3.5 hours at 42°C. Nitrocellulose filters are numbered with a soft-lead pencil. The filters are handled with gloved hands. The filters are soaked in a solution of isopropylthio-ß-*D*-glacatoside (IPTG) (10mM in distilled water) for 10 minutes. Using blunt-ended forceps, the filters are removed from the solution and allowed to dry at room temperature. The plates which have been allowed to incubate for 3.5

hours are overlaid with the nitrocellulose filters. The plate is then incubated for at least 4 hours at 37°C. Duplicate filters are prepared by removing the first filter after 4 hours and replacing it with a second numbered filter that has been impregnated with IPTG. The plate is then incubated for a further 4-6 hours at 37°C after which the second filter is removed.

Filters are peeled off the plates and immediately immersed in a large volume of TNT (10mM Tris-Cl (pH 8.0); 150mM NaCl; and 0.05% Tween 20). The filters are transferred individually to glass trays containing blocking buffer (20% fetal bovine serum in TNT). The filters are incubated for 30 minutes at room temperature.

The filters are transferred to fresh glass trays containing labeled protein diluted in blocking buffer.

Labeled protein is prepared from two sources, normal cells and tumor cells. Differentially expressed proteins can be detected by a comparison of binding of the labeled protein to immobilize antibody.

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Using the methods described above, specific peptide-phages are panned against purified recombinant target proteins such as, for example, epithelial growth factor receptor (EGF-R) and platelet-derived growth factor receptor (PDGF-R), by conventional methods. Panning can be conducted, for example, as described in the following, including the references cited in these references: Wrighton *et al.* (1996) *Science* 273:458-463; Cwirla *et al.* (1997) *Science* 276:1696-1699; Cesareni (1992) *FEBS Lett.* July 27; 307(1):66-70; Desai *et al.* (1998) *Cancer Res.* 58(11):2417-2425; Carcamo *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95(19):11146-11151; Gardsvoll *et al.* (1998) *FEBS Lett.* 431(2):170-174; Williams *et al.* (1998) *J. Immunol. Methods* 213(1):1-17; and Giersch *et al.* (1994) *Curr. Biol.* 4(2):173-174.

The first set of experiments is illustrated in Table 1. Increasing amounts of recombinant target proteins will be separated by SDS-PAGE and Western-blotted onto nitrocellulose membranes. Individual membranes are probed with peptide-phages against, for example, either EGF-R(B) or PDGF-R(C). Binding affinity and

specificity of the peptide phages to the individual target proteins is visualized by a secondary antibody against M13 phage particle linked to alkaline phosphatase. Table 1 shows that peptide-phages bind with high specificity. Affinities of specific peptide-phages to target protein receptors are expected to differ.

Table 1. Immuno blot-type experiment using specific peptide-phages against specific target protein receptors.

A. Peptide-Phage against EGF-R			
Target Protein	Recombinant 1	Recombinant 10	Protein 100 ng
EGF-R	----	+	++
PDGF-R	----	----	----

B. Peptide-Phage against PDGF-R			
Target Protein	Recombinant 1	Recombinant 10	Protein 100 ng
EGF-R	----	----	----
PDGF-R	+++	+++	+++

The second set of experiments is illustrated in Figure 1. Peptide-phages are immobilized using a monoclonal mouse-anti M13 antibody and incubated with a cell lysate or a mixture of different recombinant proteins including the target protein receptor. After extensive washing using standard techniques, the bound target protein receptor will be identified using a specific antibody against the target protein receptor linked to a detection molecule, for example alkaline phosphatase (goat antibody to target protein receptor) for detection.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. A method for identifying an antibody which binds to a differentially expressed protein, comprising the steps of:

(a) contacting labeled proteins from a first cell with a first solid support under conditions where antibodies specifically bind to proteins, wherein the solid support comprises a pattern of antibody expression clones, wherein the antibody expression clones express fusion proteins comprising single chain antibodies, wherein the single chain antibodies are immunoreactive with proteins of the first cell;

(b) determining a first pattern of immunoreactivity on the first solid support formed by the binding of labeled proteins from the first cell with the single chain antibodies expressed by the antibody expression clones;

(c) contacting labeled proteins from a second cell with a second solid support comprising the pattern of antibody expression clones;

(d) determining a second pattern of immunoreactivity on the second solid support formed by the binding of labeled proteins from the second cell with the single chain antibodies expressed by the antibody expression clones; and

(e) comparing the first pattern of immunoreactivity with the second pattern of immunoreactivity, wherein a difference between the first and second patterns of immunoreactivity identifies an antibody which binds to a protein which is differentially expressed between the first cell and the second cell.

2. The method of claim 1, wherein the first and second solid supports comprise identical patterns of antibody expression clones.

3. The method of claim 1, wherein the second solid support is the first solid support which has been treated to remove bound labeled proteins from the first cell.

4. The method of claim 1, wherein the antibody expression clones are bacteriophage.

5. The method of claim 1, wherein the antibody expression clones are bacteria.

6. The method of claim 1, wherein the solid supports comprise a pattern of beads, wherein the antibody expression clones are bound to the beads.

7. The method of claim 6, wherein the beads are reversibly attached to the solid support.

8. The method of claim 6, wherein identifying labels are attached to the beads.

9. The method of claim 1, wherein the single chain antibodies are expressed on the surface of the antibody expression clones.

10. The method of claim 1, wherein either the first or the second cell is a neoplastic cell, wherein if the first cell is a neoplastic cell then the second cell is a non-neoplastic cell, and if the second cell is a neoplastic cell then the first cell is a non-neoplastic cell.

11. The method of claim 1, wherein either the first or the second cell is a highly metastatic cell, wherein if the first cell is a highly metastatic cell then the second cell is a weakly metastatic cell, and if the second cell is a highly metastatic cell then the first cell is a weakly metastatic cell.

12. The method of claim 1, wherein either the first or the second cell is infected with a pathogen, wherein if the first cell is infected with the pathogen then the second cell is not infected with the pathogen, and if the second cell is infected with the pathogen then the first cell is not infected with the pathogen.

13. The method of claim 1, wherein either the first or the second cell is a diseased cell, wherein if the first cell is a diseased cell then the second cell is a

non-diseased cell, and if the second cell is a diseased cell then the first cell is a non-diseased cell.

14. The method of claim 1, wherein the first cell is at a first developmental stage and the second cell is at a second developmental stage.

15. The method of claim 1, wherein either the first or the second cell is exposed to an environmental factor, wherein if the first cell is exposed to the environmental factor then the second cell is not exposed to the environmental factor, and if the second cell is exposed to the environmental factor then the first cell is not exposed to the environmental factor.

16. The method of claim 1, wherein either the first or the second cell is contacted with an exogenous substance, wherein if the first cell is contacted with the exogenous substance then the second cell is not contacted with the exogenous substance, and if the second cell is contacted with the exogenous substance then the first cell is not contacted with the exogenous substance.

17. The method of claim 1, wherein the first cell originates from a first tissue type and the second cell originates from a second tissue type.

18. The method of claim 1, wherein the first cell originates from a first species and the second cell originates from a second species.

19. The method of claim 1, wherein the labeled proteins are radioactively labeled.

20. The method of claim 1, wherein the labeled proteins are biotinylated.

21. A method for identifying a gene which is differentially expressed between two cells comprising the steps of:

(a) identifying a single chain antibody which binds to a protein which is differentially expressed between two cells according to the method of claim 1;

(b) isolating the identified single chain antibody; and

(c) using the single chain antibody to screen a population of protein expression clones comprising cDNA obtained by reverse transcription of mRNA isolated from the first cell, wherein a protein expression clone which binds to the single chain antibody is identified as expressing a cDNA encoding a protein which is differentially expressed between the two cells.

22. A solid support comprising a pattern of antibody expression clones, wherein the antibody expression clones express fusion proteins comprising single chain antibodies.

23. The solid support of claim 22, wherein the single chain antibodies are immunoreactive with proteins in a non-diseased cell.

24. A kit comprising at least two solid supports according to claim 22.

25. The kit of claim 24 further comprising a viable sample of the cell population.

26. A kit for identifying a gene which is differentially expressed between a first cell and a second cell, comprising:

(a) a first solid support comprising a pattern of antibody expression clones, wherein the antibody expression clones express fusion proteins comprising single chain antibodies, wherein the single chain antibodies are immunoreactive with proteins of the first cell, wherein the first solid support can be used to identify a single chain antibody which binds to a protein which is differentially expressed between a first cell and a second cell; and

(b) a second solid support comprising a pattern of protein expression clones comprising CDNA obtained by reverse transcription of mRNA

isolated from the first cell, wherein the protein expression clones express cDNA encoding proteins of the first cell, wherein the second solid support can be screened with the identified single chain antibody to identify a protein expression clone which expresses a cDNA encoding a protein which is differentially expressed between the first cell and the second cell.

27. The kit of claim 26 further comprising a viable sample of the first cell for preparing proteins to screen the single chain antibodies.

28. A method of identifying a gene which encodes an intracellular protein which participates in a cellular response, comprising the steps of:

(a) introducing a library of nucleic acid vectors encoding single chain antibodies into a population of cells, wherein the vectors express single chain antibodies intracellularly, wherein the single chain antibodies bind to intracellular proteins of the population of cells;

(b) contacting the population of cells with an inducer of a cellular response, wherein the inducer induces the cellular response in a first subset of the population of cells but does not induce the cellular response in a second subset of the population of cells;

(c) isolating at least one nucleic acid vector encoding a single chain antibody from the second subset of the population of cells and expressing the single chain antibody; and

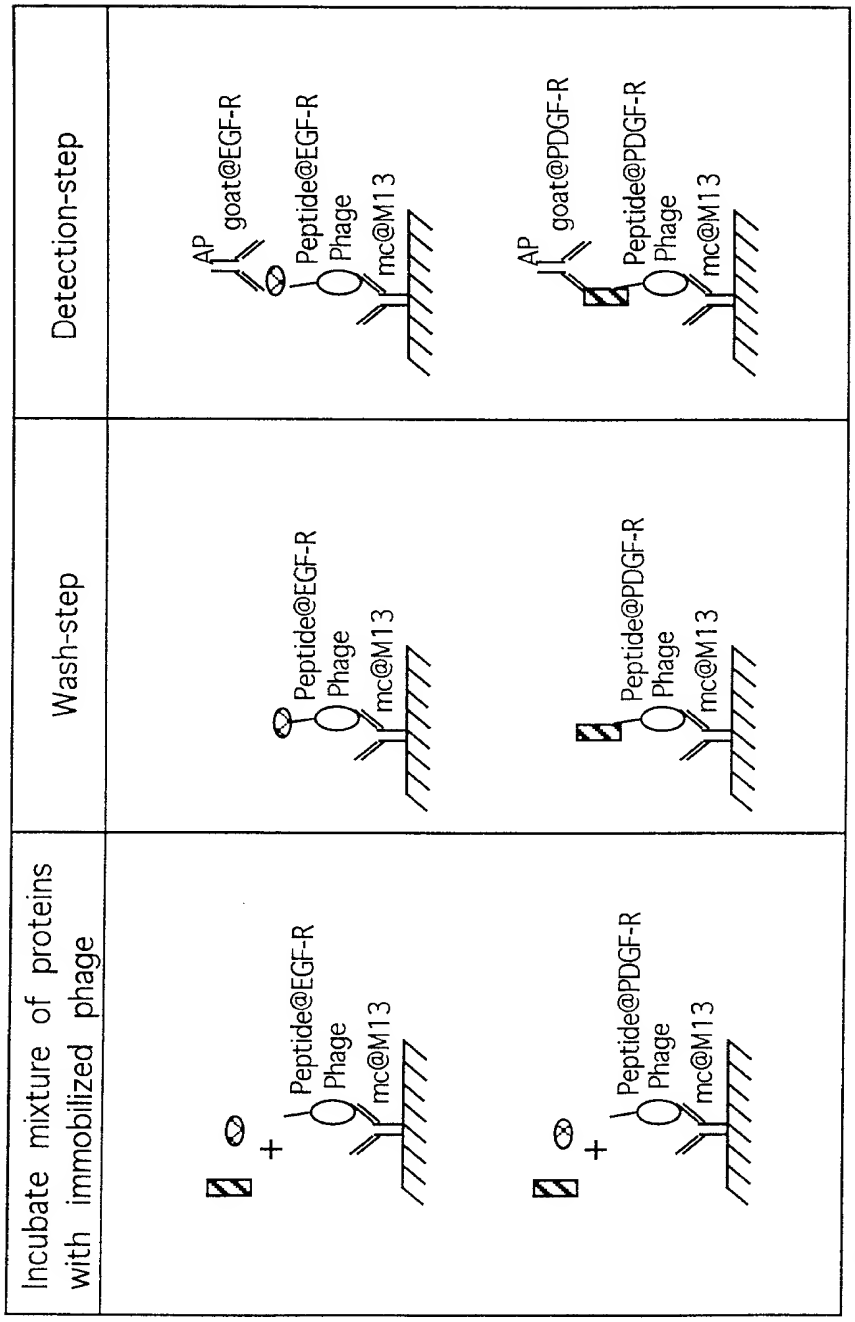
(d) using the expressed single chain antibody encoded by the isolated vector to screen a population of protein expression clones comprising cDNA obtained by reverse transcription of mRNA isolated from the population of cells, wherein a protein expression clone which binds to the expressed single chain antibody is identified as expressing a cDNA encoding an intracellular protein which is involved in the cellular response in the population of cells.

29. The method of claim 28, wherein the inducer is Fas ligand and the cellular response is apoptosis.

30. The method of claim 28, wherein the inducer is a pathogen and the cellular response is resistance to the pathogen.

31. The method of claim 28, wherein the inducer is a toxin and the cellular response is apoptosis.

Figure 1. ELISA-type experiment using specific immobilized peptide-phages to capture specific target protein receptors.



INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/US 98/19425

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 1999	24/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Routledge, B

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